

1-1-1991

## Immunohistochemical Techniques Applied to Raw and Mildly Heat Treated Meat Systems

B. Egelandsdal

*Norwegian Food Research Institute*

S. Kidman

*The Swedish Food Research Institute*

A.-M. Hermansson

*The Swedish Food Research Institute*

Follow this and additional works at: <https://digitalcommons.usu.edu/foodmicrostructure>

---

### Recommended Citation

Egelandsdal, B.; Kidman, S.; and Hermansson, A.-M. (1991) "Immunohistochemical Techniques Applied to Raw and Mildly Heat Treated Meat Systems," *Food Structure*: Vol. 10 : No. 4 , Article 4.

Available at: <https://digitalcommons.usu.edu/foodmicrostructure/vol10/iss4/4>

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Food Structure by an authorized administrator of DigitalCommons@USU. For more information, please contact [digitalcommons@usu.edu](mailto:digitalcommons@usu.edu).





## IMMUNOHISTOCHEMICAL TECHNIQUES APPLIED TO RAW AND MILDLY HEAT TREATED MEAT SYSTEMS

B. Egelanddal<sup>1</sup>, S. Kidman<sup>2</sup> and A.-M., Hermansson<sup>2</sup>

<sup>1</sup>MATFORSK, Norwegian Food Research Institute, Oslovn. 1, N-1430-Ås, Norway and

<sup>2</sup>SIK, The Swedish Food Research Institute, P. O. Box 5401, S-402 29 Gothenburg, Sweden

### Abstract

Immunohistology was performed with six commercially available antibodies directed against myosins, actins and collagens. The corresponding antigens, appearing on the surface of cryo-sections from meat and meat products heat treated to different end temperatures, were visualized using these antibodies. The meat and meat products were heated from 20 °C to 80 °C. At 80 °C the meat systems were devoid of thermal transitions as judged from differential scanning calorimetric measurements.

Our results showed that although reduced binding was the case for systems heated above 60 °C, the signals from the antibody labelling was still sufficiently strong to provide information about specific antigens in meat systems heated to 70-80 °C. Antibodies with a high initial affinity bound to the their respective antigens after the latter had been heated to a few degrees above their denaturation temperature as detected in a scanning calorimeter. This investigation points to the possibility of finding sufficiently good commercial antibodies to perform immunohistology on meat products heated to temperatures between 70-80 °C. This is important as many commercial meat products are heated to end temperatures in this range. Several examples of the labelling intensity obtained on heated meat and meat products are given. In addition, an example using double labelling with antibodies to collagen III and an antibody to slow myosin in a food product heated to 75 °C is given. Problems related to non-specific staining and to non-specific effects of heat treatment are also briefly discussed.

**Key words:** Immunohistology, cryo-sections, light microscopy, meat, comminution, heating, salting, myosin, actin, collagen.

### Introduction

In order to study technologically important processes such as storage, mixing, curing and heating, microscopy is useful as it is possible to detect the location of one specific component in the tissue or product examined. Several histological techniques have been developed for the location of actin (Tang *et al.*, 1989), myosin (Brooke and Kaiser, 1970; Stevens, 1982), collagen (Sweat *et al.*, 1964) and elastin (Keith *et al.*, 1977; Romeis, 1989). These techniques rely, in general, on the affinity of different low molecular weight dyes and substrates for particular molecules or enzymes, respectively. Sometimes the contrast is increased by adding a second step before staining; such as partial denaturation in the case of adenosine triphosphate (ATP)-ase staining for myosin. Some of these techniques are difficult to perform and the results difficult to interpret (Fakan and Saskova, 1982; Pierard, 1989). Techniques based on immunohistochemical principles might overcome some of these problems. For diagnostic and research purposes in medicine and plant biology, immunohistochemical identification has been used for some time because of its high specificity. In food diagnostics and especially, in the study of the functional performance of food ingredients, this technique is still quite young.

The number of commercially available poly- and monoclonal antibodies directed against mammalian proteins, pathogenic bacteria and viruses is steadily increasing. Some of these antibodies, which are mostly raised against human antigens, would be expected to possess adequate cross reactivity with meat proteins. This makes it attractive to substitute less specific techniques with immunological techniques even for scientists with limited experience in immunology.

Only one paper (Zijderveld and Koolmees, 1990) has so far been devoted to the use of immunohistology for functional studies of components in real meat products. Their paper is rather discouraging to potential new users. They reported weak binding to the majority of their frozen hydrated sections taken from unheated and heated comminuted meats. Better results were, however, obtained on paraffin sections. The fixation

---

Initial paper received May 30, 1991  
Manuscript received September 30, 1991  
Direct inquiries to B. Egelanddal  
Telephone number: 47 9 97 0100  
FAX number: 47 9 97 0333

---



procedure was suggested as a partial explanation of the reported differences between paraffin and frozen sections. Zijderveld and Koolmees (1990) did not present any illustrations of their immunolabelling signals.

Our approach is quite similar to that of Zijderveld and Koolmees (1990), *i.e.*, we want to use this technique to extract information about the functional performance of meat components. We have used six different antibodies raised against human actins, fast and slow myosin, collagen III and IV. Collagen III is distributed among all three levels of connective tissue while type IV is located exclusively in the *endomysial* basement membrane (Bailey and Light, 1989). These antibodies were bound to cryo-sections of heated meat products. The signal of the labelling is shown and discussed. The most abundant protein components present in meat can be monitored using differential scanning calorimetry (DSC, Stabursvik and Martens, 1980). Using this technique the changes in antibody reactivity with temperature were related to the progress in the denaturation of antigens.

## Materials and Methods

### Meat samples

Three different types of muscle preparations were investigated.

**System 1:** The bovine muscles *masseter*, *infraspinatus* and *cutaneus trunci* were taken on the slaughter-line, immediately put on ice and frozen in freon cooled in liquid nitrogen 6-20 hours *post mortem*. These three muscles were chosen on the basis of their wide variation in fiber type composition (Young and Davey 1981; Rao and Gault 1989; Meyer and Egelanddal, 1992). *Masseter* is a pure type I or slow red muscle. *Cutaneus trunci* is largely a type II muscle consisting of about 70% fast white (IIB) fibers, while *infraspinatus* is a mixed muscle with significant variation in composition across the muscle. The ultimate pH values for these muscles were 5.95, 5.80 and 5.50, respectively.

**System 2:** The above muscles, chilled at 11-14 °C for 24 hours, were trimmed of visible fat and connective tissue and cut into small pieces (diameter 5 mm). A portion of the small pieces was chopped 3 x 5 sec in a Moulinex Chopper type 320. Forty ml per 100 g meat of a saline solution were then added to whole and chopped meats followed by storage for 2 days in order to obtain an even distribution of the added NaCl in the intact meat. The majority of the samples were then heated to different end temperatures using a heating rate of 1 °C/min and finally frozen.

**System 3:** A commercial sample of pork which had been ground, presalted and heated to a core temperature of 75 °C was used. This system represented a real food

product and details about the preprocessing conditions and muscles types are unknown.

All meat systems were stored at -80 °C prior to sectioning and light microscopy.

### Primary antibodies directed against actins, myosins and collagens

One or two batches of each of the primary antibodies were used.

**Anti-actins:** Monoclonal antibodies to rabbit and human actins (Mon 4001, IgM and MU090-UC, IgG) were purchased from Sanbio BV (Netherlands) and from Biogenex Laboratories (USA), respectively. These two antibodies were reported to react with  $\alpha$ -actins from several species although neither beef or pork were specifically mentioned.

**Anti-myosins:** Two monoclonal antimyosins (RPN 1168 and RPN 1167, both IgM) from Amersham Corporation (USA) against human anti-slow myosin and anti-fast myosin were reported to react with myosins from several species, however, beef or pork myosins were not specifically mentioned.

**Anti-collagens:** Two goat polyclonal antibodies against human collagens (Cat no 1330-01 and 1340-01) from Southern Biotechnology (USA) were chosen. These antibodies against collagens are sold as specific also for cow collagen III and IV.

### Secondary antibodies

Biotinylated antibodies were purchased from Sigma Chemical Co., USA (Goat anti-mouse IgM, biotinylated, product no B-9265 and goat anti-mouse polyvalent immunoglobulins, biotinylated, product no B-2016); Amersham International, USA (Goat anti-mouse IgG, biotinylated, prod. no RPN 1180); and from The Binding Site Ltd. (Donkey anti-goat IgG, biotinylated, code HB 240) for use in combination with antibodies against myosins, actins and collagens.

### Blocking reagents

Blocking reagents were bovine serum albumin (crystalline grade, different producers), goat serum or donkey serum (Seralab Ltd., England).

### Detection systems

Streptavidin bound fluorescein isothiocyanate (FITC, RPN 1232) was purchased from Amersham International (USA). Peroxidase ABC kit (PK-4000) and Phosphatase AP kit (SK-5100) were purchased from Vector Laboratories, USA.

### Substrates

Chlornaphtol tablets (No. C 6788, substrate for peroxidase) were purchased from Sigma Chemical Co., USA. As substrate for phosphatase, kit I, product no



SK 5100, from Vector Laboratories, USA, was used.

#### Other chemicals

Rhodamine-phalloidin was purchased from Molecular Probes (Junction City, Oregon, USA), ATP from Sigma Chemical Co. (USA).

#### Myosin ATP-ase staining (fiber typing)

This was performed essentially in accordance with Brooke and Kaiser (1970). Preincubation was for 4 minutes at pH 4.3. Under these conditions slow myosin stains dark.

#### Rhodamine-phalloidin staining for actin

This was performed in accordance with Tang *et al.* (1989) using 3% paraformaldehyde and acetone at -20 °C as fixative.

#### Immunolabelling and staining

The primary antibodies were used diluted 1:10 (Sanbio's antibody against actin), 1:200 (Biogenex' antibody against actin), 1:10 (Amersham's antibody against myosins), and 1:400 (from originally 0.5 mg/ml for Southern Biotechnology's antibodies against collagens). The diluent was normally phosphate buffered saline (PBS) containing 1% bovine serum albumin. Appropriate dilutions were established using a test series of different dilutions. Incubation time was usually 1 hour at room temperature or at 37 °C. In those cases where a weak signal was seen after 1 hour incubation, and the intensity of labelling was not to be compared with other systems incubation overnight at 4 °C was practiced for the primary antibody. Negative controls were always included. The secondary antibodies were diluted as follows: 1:50 for the anti-mouse antibodies and 1:400 for the anti-goat antibodies. The blocking reagent (5% serum plus 5% crystalline bovine serum albumin in PBS, or in 0.25 M NaCl, pH 7.5, for antibodies against fast myosin) was applied for 30 minutes on the sections both before the primary antibody and the secondary antibody were applied. Following incubations with antibodies, the sections were thoroughly rinsed with PBS. The rest of the labelling procedure was in accordance with common routines (Amersham International plc.: The biotin-streptavidin system, pp 11-20; Bourne, 1983). Double labelling was performed as two consecutive single labellings. The samples labelled with FITC were mounted in Citifluor (Citifluor Ltd., London, UK). Other objects were mounted in a gum arabic/sucrose solution.

#### Light microscopy

Only cryo-sections (~ 5 µm thick) were examined. As a routine no fixatives were used, although the effect of some fixatives like paraformaldehyde, acetone, ethanol and heating were tested. A NIKON microphot-

FXA microscope was employed. The instrument had equipment for epifluorescence, and had a HBO-100 W/2 lamp for fluorescence and a Hg 100 W lamp for bright field microscopy. Filter B2 was used for the fluorochrome FITC and filter ND for the rhodamine-phalloidin labelling. The objects labelled with fluorochrome were photographed with 400 and 125 ASA black and white films as well as 400 and 160 ASA color films. Objects labelled with enzymes were photographed with 125 ASA black and white or 50 and 160 ASA color films.

**Quantitative fluorescence microscopy:** For some samples labelled with FITC, the light emitted was measured. This was performed as soon as possible after labelling and care was taken to keep the slides free from exposure to any light prior to measurements. Ten to fifteen readings of the light emitted by the fluorochrome upon illumination of the muscle fiber were made on different areas within each object. An objective, which magnified the samples ten times, was used. The autofluorescence of the meat sample (non-labelled reference) was recorded and subtracted from labelled samples. The autofluorescence for meat was about 1 mlx depending on the type of muscle involved and the specific treatment given to the muscle. Some areas of the meat also had a higher autofluorescence than others, however, readings were taken from relevant areas.

#### Differential scanning calorimetry (DSC)

Calorimetric measurements were performed with a Setaram Micro DSC-batch and flow calorimeter (Setaram, Lyon, France). Heating rate was 1 °C/min. The sample size used was 1 gram. In order to speed up the effect of salt on system 1, chopped meat with saline added, was used for calorimetric investigations.

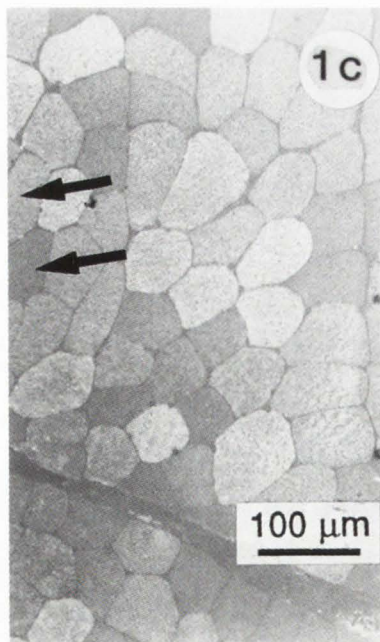
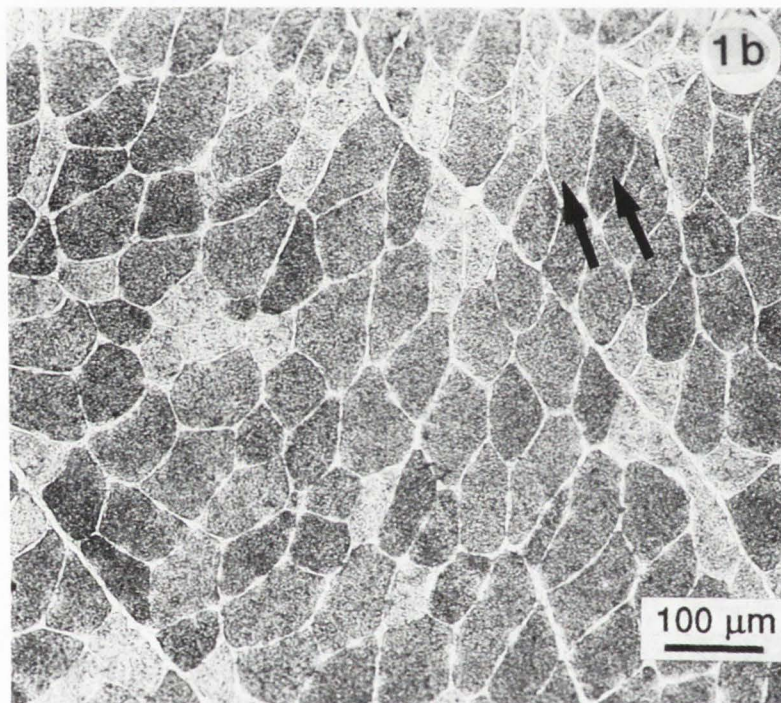
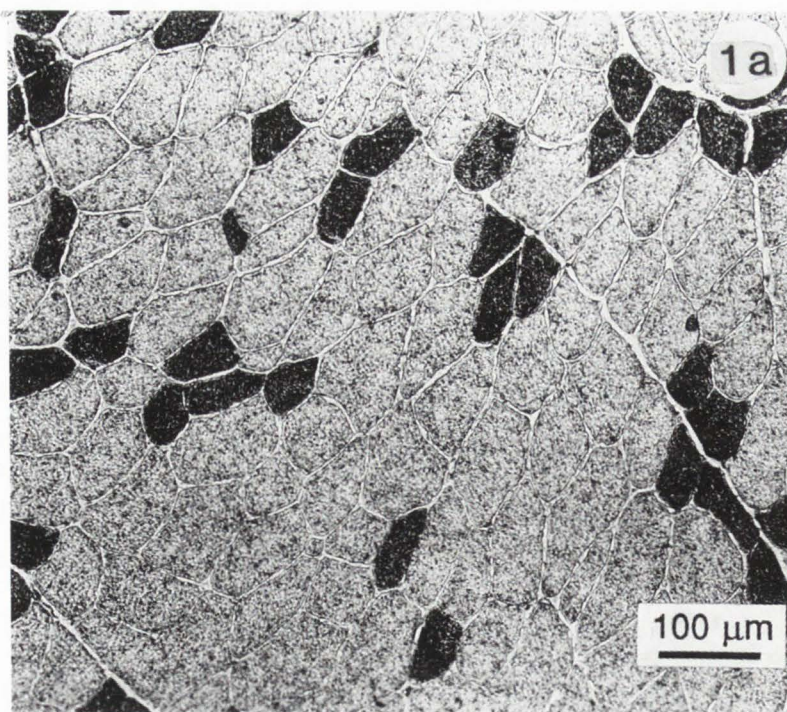
### Results

The results below relate to the general use of antibodies raised against muscle proteins in meat systems. Figures 1 and 2 are devoted to the process of verifying correct labelling. This means that antibodies raised against human antigens should have sufficiently strong binding for the corresponding antigen in beef or pork tissue, *i.e.*, good species cross reactivity, and not bind to other molecules contained in those tissues. The changes in labelling density caused by denaturation of the antigens chosen, are shown in Figures 3-8. Eventually the choice of fixative is reported on.

#### Species cross reactivity

Species cross reactivity for the monoclonal antibodies against myosin and actin was checked by comparing the immunolabelling with staining for ATP-ase activity and binding to phalloidin, respectively (Figures 1 and 2).





**Figure 1 (at left).** Comparison of immunolabelling with antibodies against fast myosin (a), with immunolabelling against slow myosin (b), as well as comparison of immunolabelling with antibodies against slow myosin (c), with ATP-ase staining (d) using *infraspinatus* (system 1). The objects in panels (b) and (c) were heat treated to 80 °C before labelling. The labelling was detected with alkaline phosphatase (dark for positive reaction) in panels (a) and (b). Panel (c) was labelled with FITC (dark for positive reaction). The arrows in panels (b) and (c) point to two fibers of different shades of darker grey revealed by antibodies against slow myosin. The arrows in panel (d) point to two different shades of grey detected with the ATP-ase staining.

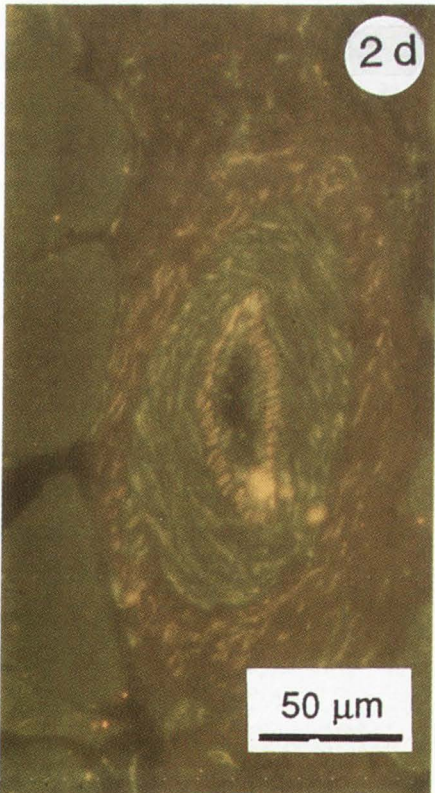
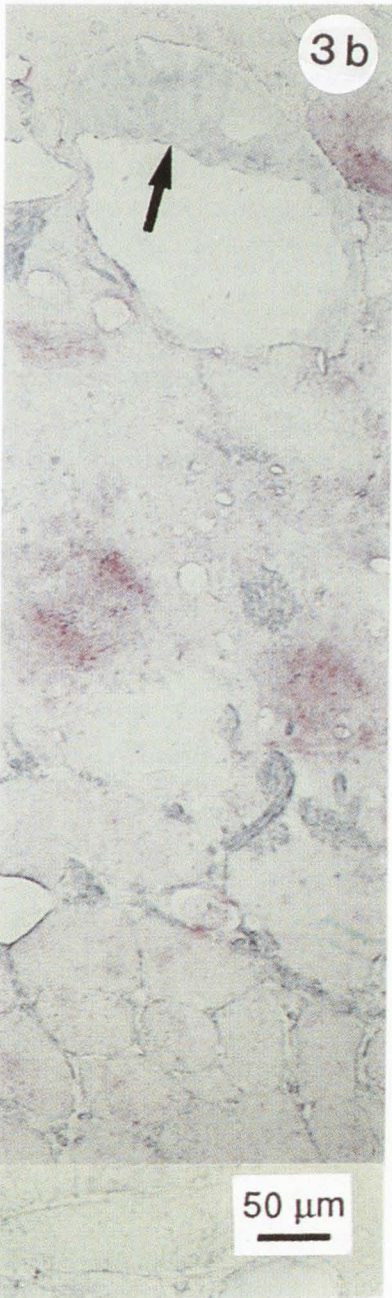
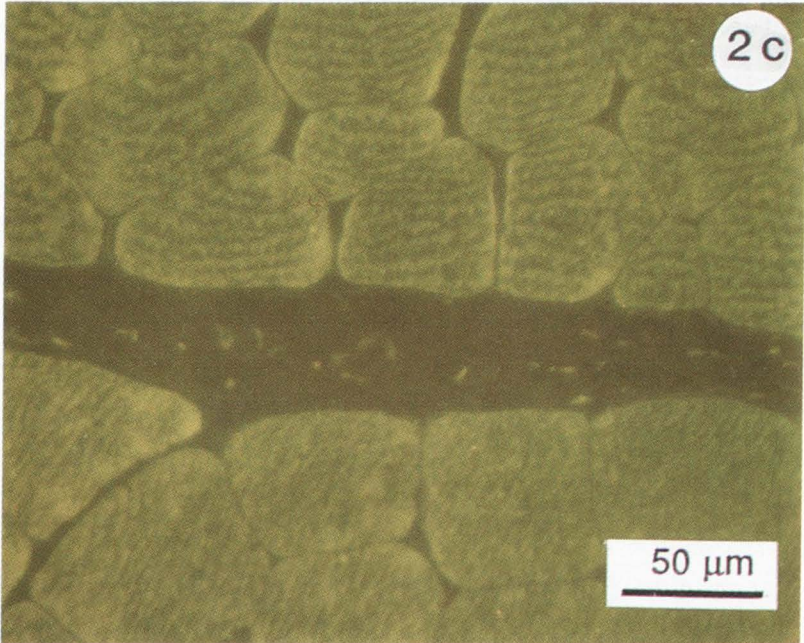
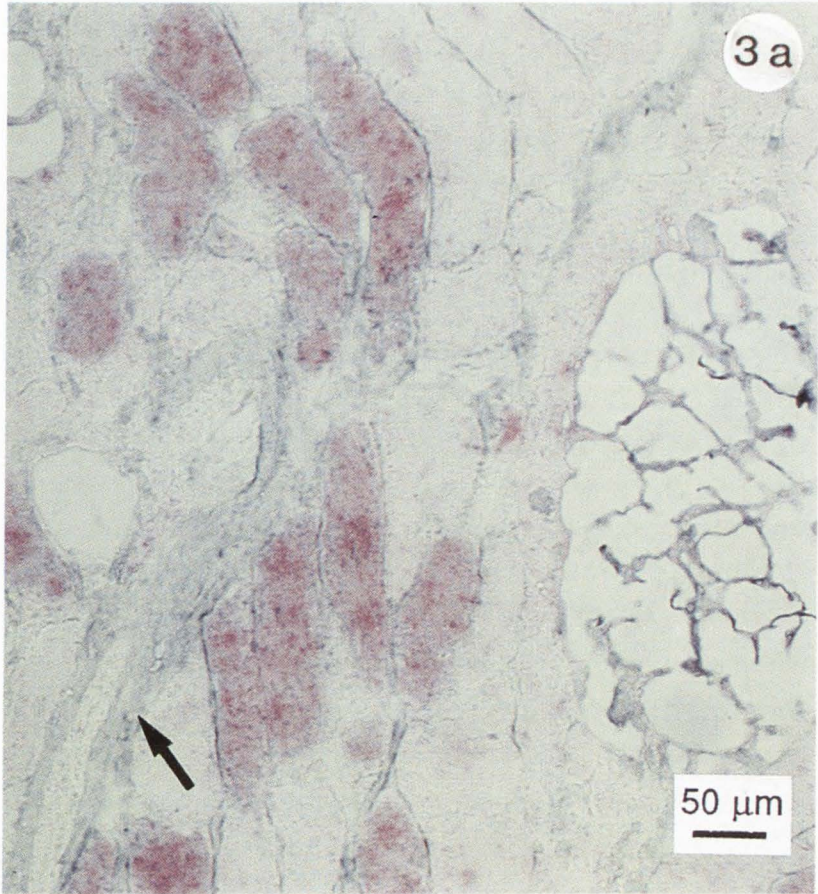
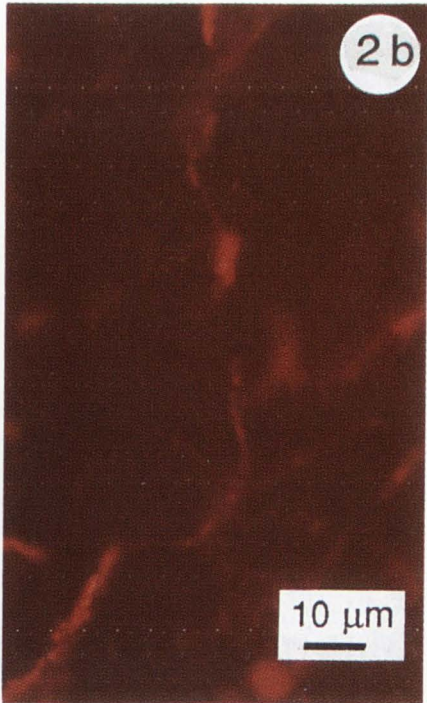
**Figure 2 (facing page, left).** Labelling for actin using rhodamine-phalloidin (panels a and b) and with antibodies against actins (panels c, d, and e, detection system FITC). For the section in panel (e) Biogenex' antibody against actin was used. Raw *infraspinatus* (panels a-c) and *masseter* (panels d and e), both system 1, were used. The arrow in panel (a) points to a blood vessel.

**Figure 3 (facing page, right).** Comminuted, presalted (2% NaCl) pork meat (system 3) heat treated to 75 °C and labelled with antibodies against slow myosin and collagen III (panels a and b). Panel (c) shows the same system labelled for collagen III only. The objects were incubated with the primary antibodies overnight (at 4 °C). The background for the double labelling is shown as the lower part of panel (b). Detection systems: alkaline phosphatase for slow myosin and peroxidase for collagen III. The photographs have been taken using a NCB11 filter which amplifies the weaker signal from collagen III using peroxidase as detection system, but deteriorates somewhat the signal from slow myosin detected with alkaline phosphatase. The arrow in panel (a) points to stained *perimysium* or *epimysium*. The arrow in panel (b) points to positively stained material which is suspected of being heat melted collagen III.

For the antibodies against myosins the presence of regularly spaced lines indicated A-bands (not shown), and the agreement in the immunolabelling pattern with the ATP-ase staining seen in Figures 1c and 1d indicated correct specificity. Figures 1b-1d show that although the differentiation was larger between type I and II fibers for both histological stains, some differentiation within fibers, indicated with arrows, was also found.

Correct binding of the antibodies against actins is assumed because of the regular pattern of labelled lines appearing along the myofibril (Figure 4c) in addition to the presence of the characteristic staining pattern for the smooth muscle around the blood vessel, indicated by







arrows in Figures 2a and 4a. Finally, stained spots in the connective tissue were found for both the antibody staining and for the rhodamine-phalloidin staining seen in Figures 2a-2e. The antibody against actin from Biogenex showed the greater affinity for  $\alpha$ -actin present in the smooth muscle of blood vessels (Figure 2e), while Sanbio's antibody against actin seemed to bind equally strong to all available  $\alpha$ -actins (Figures 2c and 2d). The spots/filaments appearing in the connective tissue for this labelled system are discussed below.

The polyclonal antibodies against collagens are sold as specific for cow collagens. In addition, they have been found to react with pig collagens (H.A. Hansson, University of Gothenburg, Sweden, Private communication) and further investigations on specificity were therefore deemed superfluous.

### Non-specific Staining / Cross reactions

For the six antibodies examined, different "non-specific" staining patterns were found. "Non-specific" staining was in most cases related to incomplete discrimination between isozymes. This means that the antibody against slow myosin also reacted somewhat with fast myosin since the fast fibers were shaded fair grey in Figure 1c or dark grey as the arrows indicate in Figure 1a. Antibodies against actins reacted with several genetic variants of  $\alpha$ -actins, *i.e.*, myofibrillar  $\alpha$ -actin (Figure 4c), vascular  $\alpha$ -actin (Figure 4c) and  $\alpha$ -actin present in the *perimysium* (Figure 2c). Antibodies against collagen IV reacted with collagen III and/or other collagens (Figure 5) since there was some staining of the *perimysium* where collagen IV is not expected to be present (Bailey and Light, 1989). This type of cross reactivity is a problem recognized by the producers and pointed out in their data sheets.

For the antibody against fast myosin some real non-specific binding to the connective tissue was present in PBS (results not shown) and the ionic strength had to be increased in order to reduce the nonspecific binding.

Sanbio's antibody bound to spots/filaments in the connective tissue (Figure 2c). These spots/filaments could not be eliminated by changing the labelling conditions, and were not always compatible with respect to size with the results obtained with the rhodamine-phalloidin labelling seen in Figures 2a and 2b. The rhodamine-phalloidin labelling revealed more and somewhat longer filaments in the connective tissue.

### Thermal Stability of Myosin, Collagen and Actin

The resulting thermograms for the muscle *masseter* containing different levels of salt are shown in Figure 6. Identification of the protein components of interest is based on previous results reported by Stabursvik and Martens (1980), Rochdi *et al.* (1985), Bernal and Stanley (1987), and Egelanddal *et al.* (submitted). The

**Figure 4 (facing page, left).** *Masseter* (system 2) heat treated to 70 °C with no (a) and 2.5% NaCl (panels b and c) added and labelled with Biogenex' antibody against actin. The objects were incubated with the primary antibody overnight. Panel (d) gives a semi-quantitative picture of the illumination from the FITC labelled objects of different ionic strengths (*masseter* = M, and *cutaneus trunci* = C). The illumination was measured on the muscle fiber. The error bars give 2 x standard error of the mean ( $n \geq 30$ ). The arrow in panel (a) points to a blood vessel.

**Figure 5 (facing page, right).** *Masseter* (system 2) labelled for anti-collagen IV: a) unheated, b) 60 °C, c) 70 °C. The sections were not from the same meat sample. The detection system was peroxidase. The arrow in panel (a) points to labelled *perimysium*.

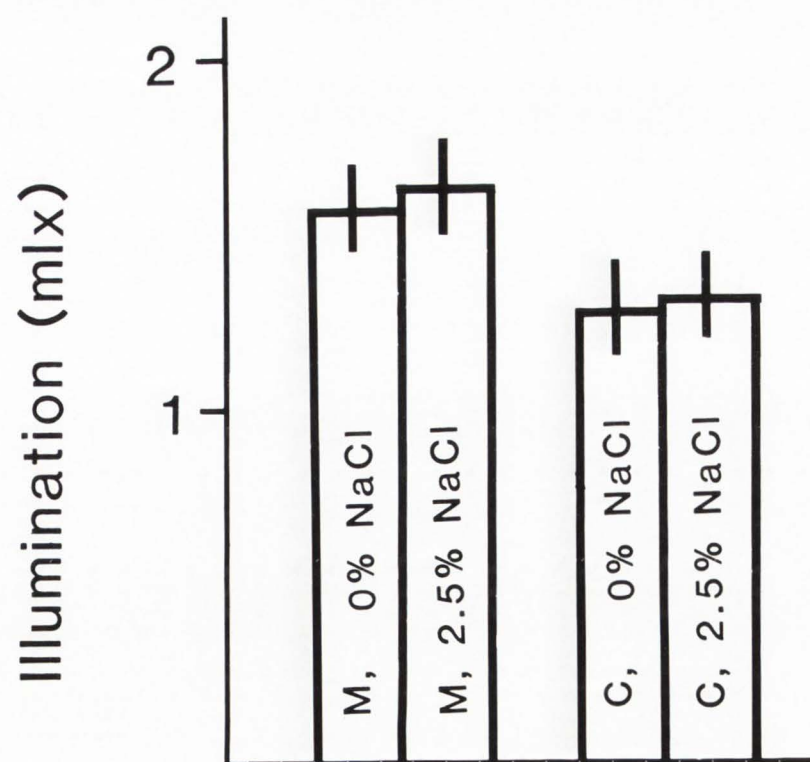
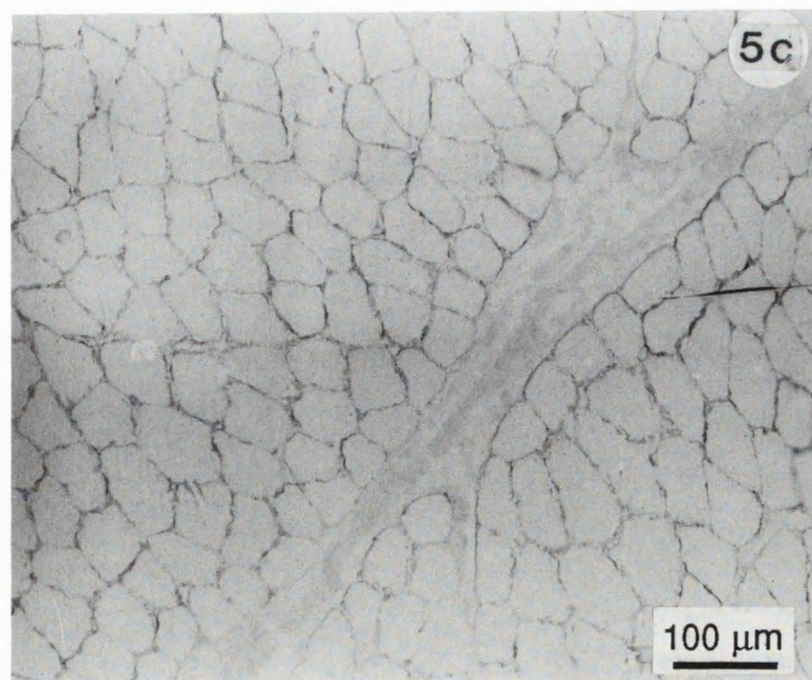
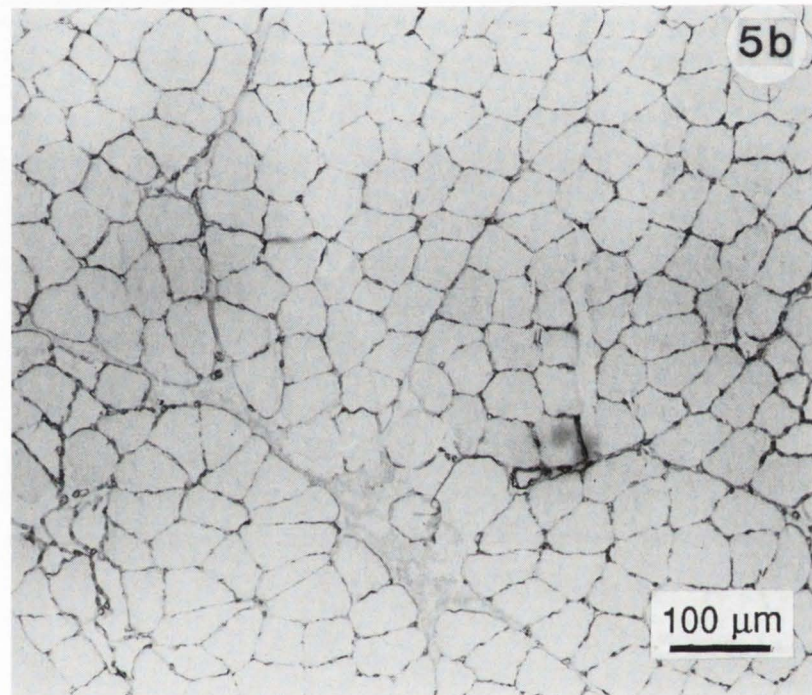
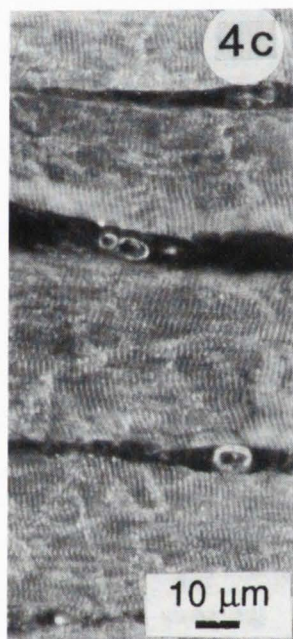
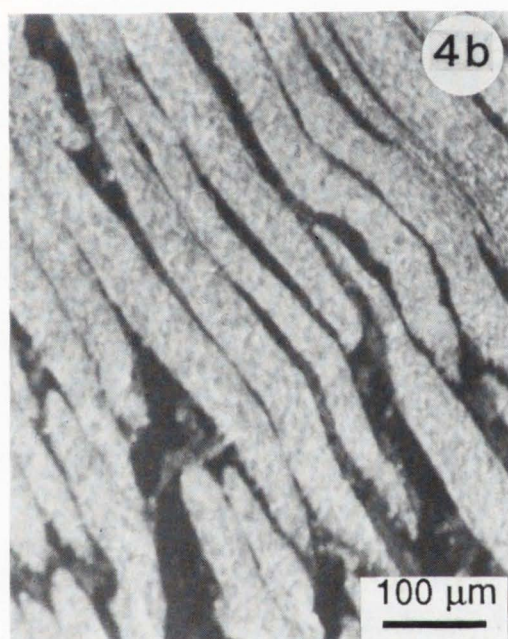
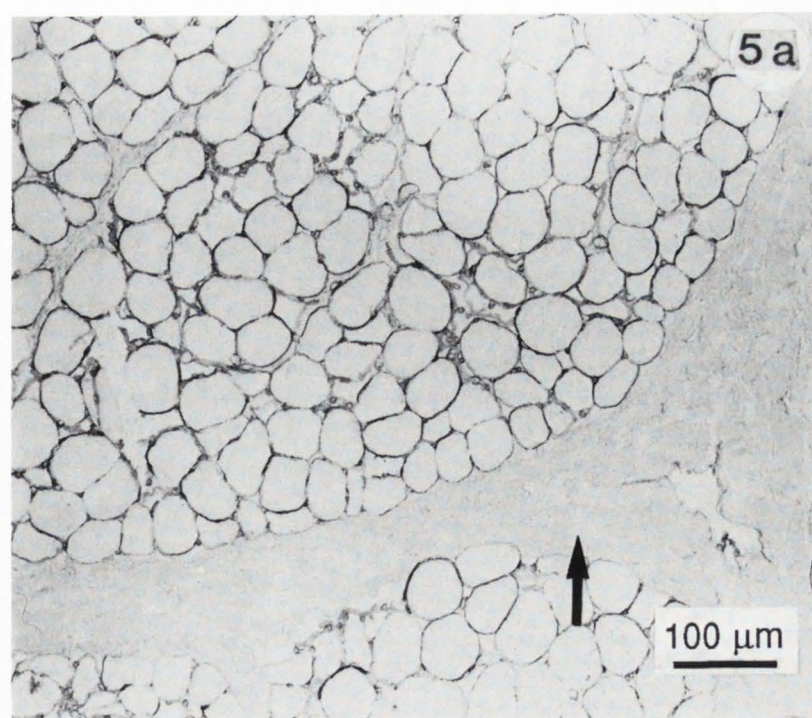
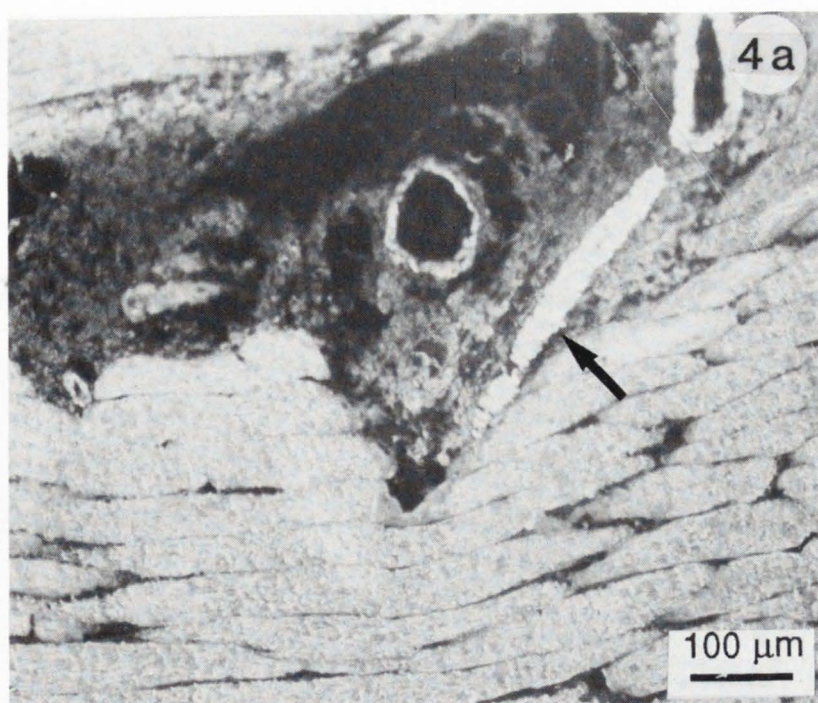
starting temperature of denaturation has been defined, for each component identified, similarly to the ending temperature of denaturation ( $T_{\text{end}}$ , see Figure 6). *Endomysial* collagen becomes thermally denatured between 50 °C and 70 °C. Temperatures around 70 °C cause rapid denaturation of actin at sufficiently high salt concentrations (Figure 6). Meat heated without and with 1% and 2.5% NaCl added is therefore denatured with respect to the three major antigens at 80 °C, 76 °C and 72 °C, respectively, as estimated from  $T_{\text{end}}$  in Figure 6.

### Binding of antibodies to meat heat treated to different end temperatures

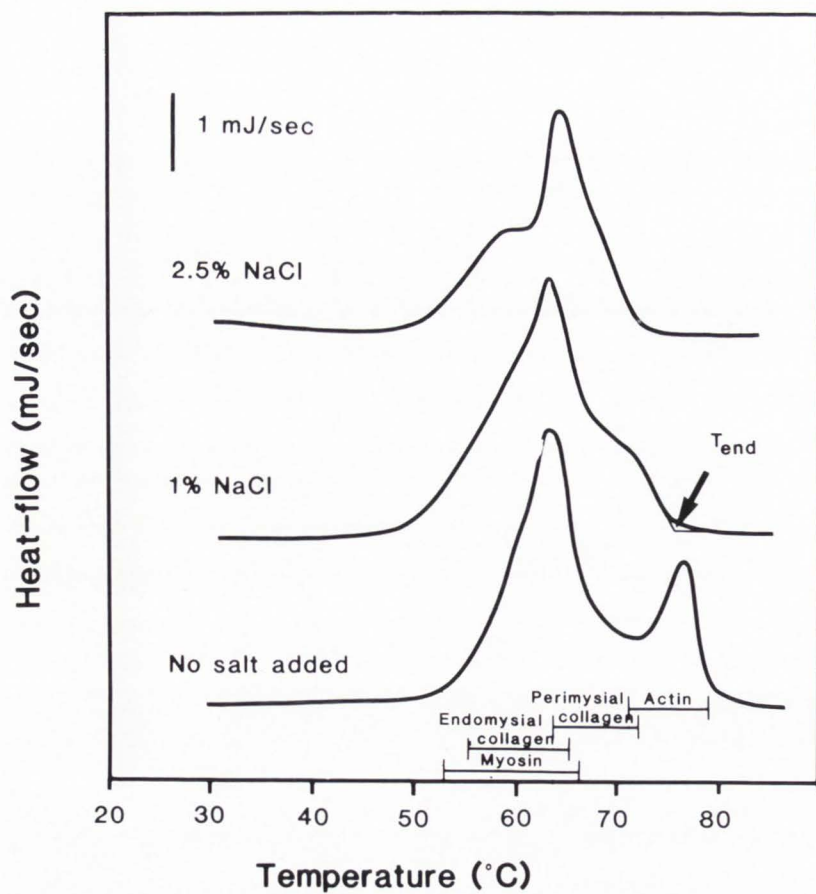
Figures 5 and 7 show *masseter* heated to different end temperatures and labelled with antibodies against collagen IV and III, respectively. Figure 5 shows that at 60 °C some changes have taken place in collagen IV, but the binding to the *endomysium* is still good. At 70 °C the labelling was weak, but still detectable. At 70 °C a partially denatured system with respect to collagen III is seen in Figure 7, panel b. Again the labelling is good especially to the *perimysium*, but also to some areas of the *endomysium*. At 80 °C the antigen is denatured and the labelling is weaker, but still positive. Labelled *endomysium* appears in lumps or fragments, indicated by arrow, and there is no labelled, intact *endomysium* surrounding the muscle fiber.

Figure 8 shows the intensity of light emitted from fresh meat labelled with antibodies against actins and myosins as a function of temperature. Figure 8a shows that both antibodies against actin bind more weakly to actin if the system has been heated to temperatures higher than 50 °C. Under standard labelling conditions (see **Materials and Methods**), labelling with Sanbio's antibody against actin at temperatures  $\geq 70$  °C and with Biogenex' antibody against actin for temperatures  $\geq 80$  °C, represents borderline cases, *i.e.*, about 1 mlx,

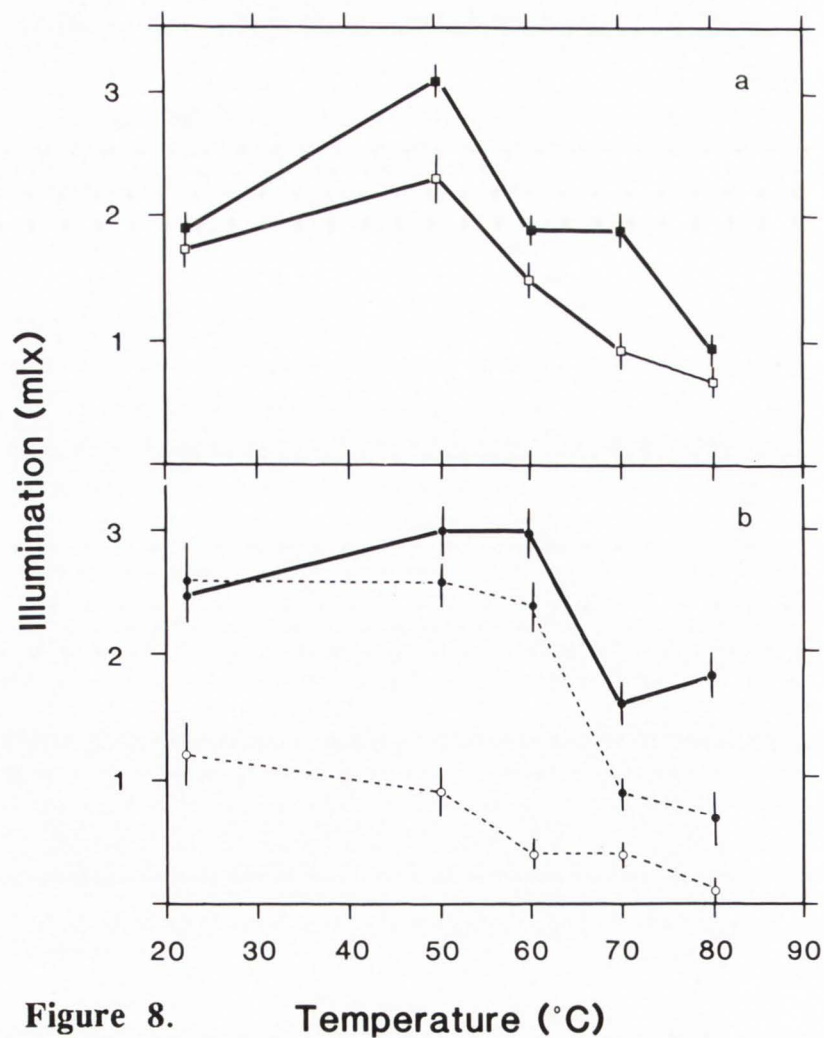




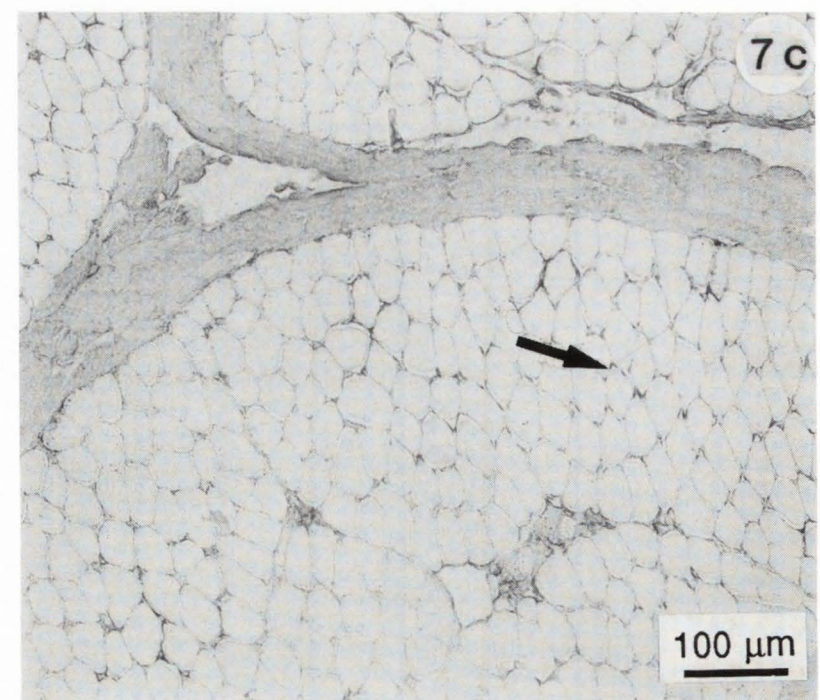
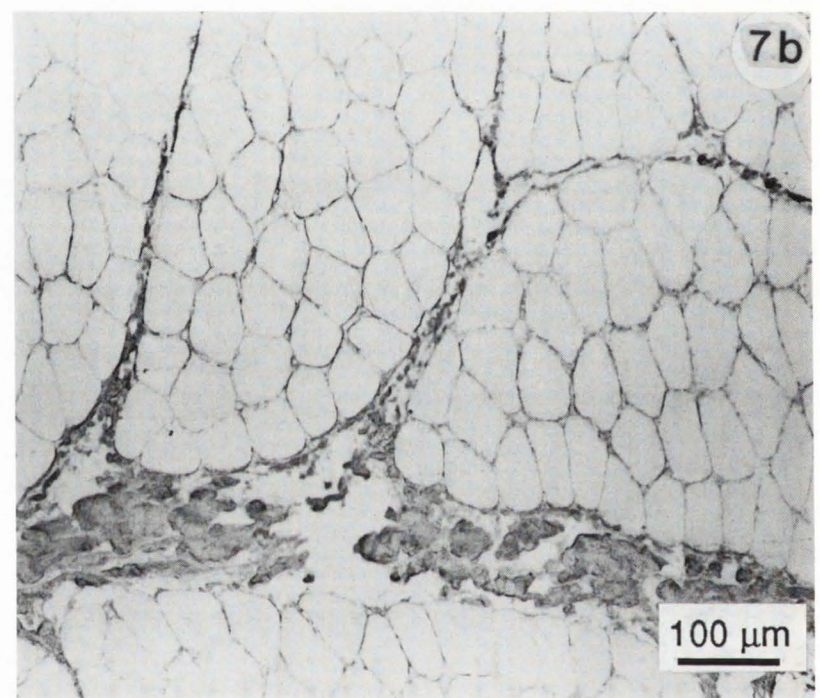
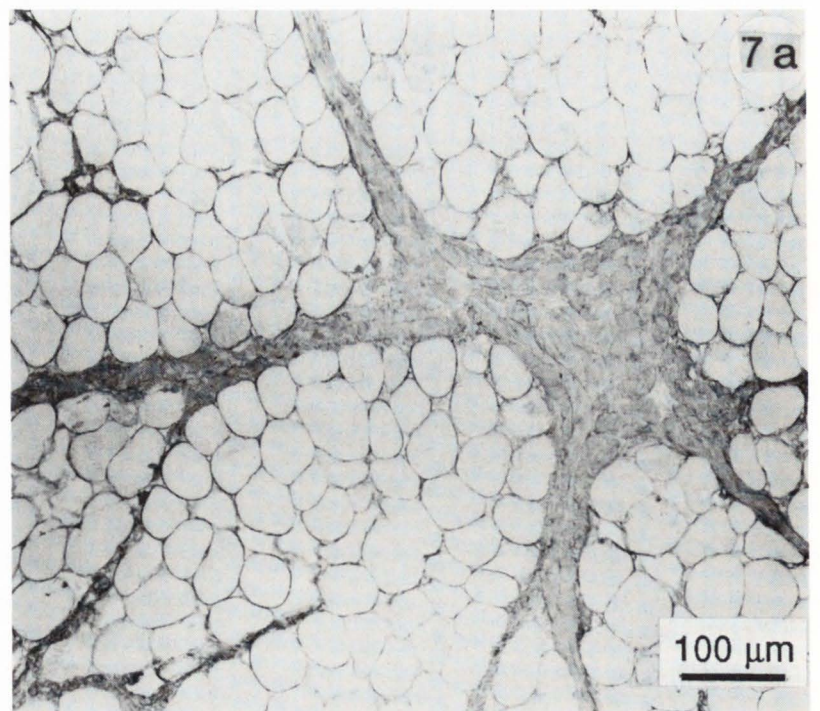




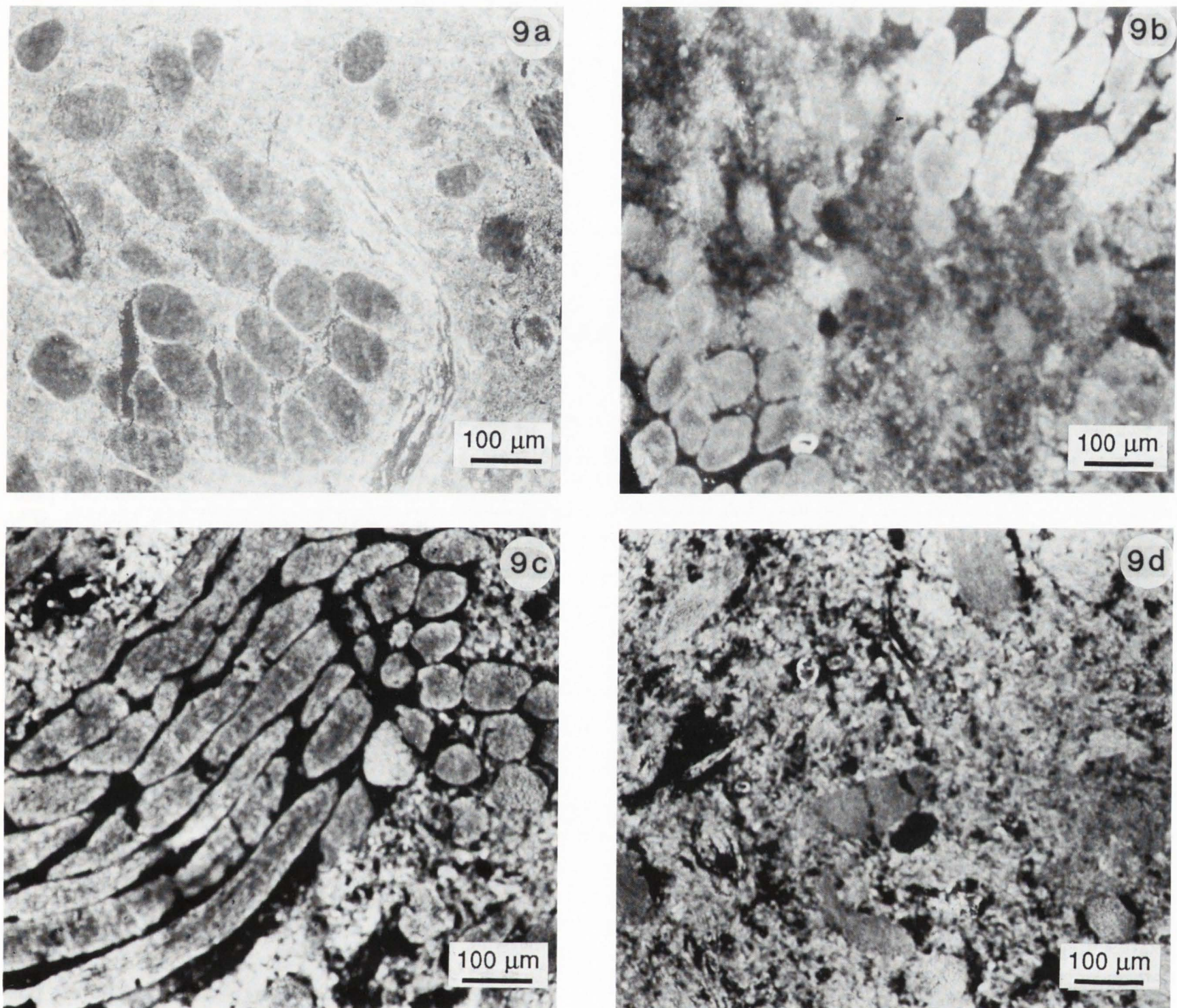
**Figure 6.** Differential scanning calorimeter thermograms (heating rate 1 °C/min) of *masseter* (system 1) containing different levels of salt (NaCl). The temperature ranges given for myosin, endomysial and perimysial collagens as well as actin indicate where the denaturation starts and ends ( $T_{end}$ ) in the absence of salt.



**Figure 8.** Temperature (°C)







**Figure 9.** Comminuted, raw *masseter* (system 2) labelled with antibodies against slow myosin (panels a and c) and actin (Biogenex, panels b and d). Panels (c) and (d) were fixed with 4% paraformaldehyde and dehydrated in ethanol starting with 30% ethanol at 0 °C and further dehydrated at -20 °C. Intact muscle appears darker in panels (a) and (c). Detection system: FITC.

---

**Figure 7 (facing page, right).** *Masseter* (system 2) labelled for anti-collagen III: a) unheated, b) 70 °C, and c) 80 °C. The sections were not from the same meat sample. The detection system was peroxidase. The arrow in panel (c) points to positions with remaining fragments of *endomysium*.

**Figure 8 (facing page, bottom left).** Illumination from FITC as a function of temperature for *masseter* (—, system 1) and *cutaneus trunci* (- - -, system 1) labelled with antibodies against actins (Biogenex = ■, Sanbio = □, panel a), slow myosin (●, panel b) and fast myosin (○, panel b). Light density below 1 mlx was experienced as too low for photographing, identification, etc. The serial cryo-sections were heat treated on the slides submerged in pH-adjusted PBS in order to insure that the same surface was available for labelling at each temperature. The bars give  $\pm$  standard deviation (n = 5-6).



with respect to the intensity of the signal from the fluorochrome. Figure 8b shows that for the antibody against fast myosin labelling is difficult above 50 °C. For the antibody against slow myosin heating above 60 °C leads to a significant fall in binding. The antibody against slow myosin can nevertheless be adequately bound to *masseter* heated to 80 °C.

Figure 4 shows *masseter* containing no added salt (panel a) and 2.5% added salt (panels b-c), heated to 70 °C and then labelled with antibody against actin. The intensity of the labelling for the antibody against actin seems very little affected by the fact that in one case the labelling is performed on essentially non-denatured actin and in the other case on practically completely denatured actin. A semiquantitative illustration of the effect of actin denaturation on binding is presented in Figure 4d for both *masseter* and *cutaneus trunci*.

Figure 3 shows meat system 3, a hamburger, which has been double labelled with red and blue for slow myosin and collagen III, respectively. Slow myosin is easily seen but collagen III only clearly when it appears in greater flakes presumably originating from *perimysium* or *epimysium* as indicated by the arrow shown in Figure 3a. Figure 3c shows system 3 single labelled for collagen III. In Figures 3a and 3b collagen III was the second antigen to be labelled. It is apparent that somewhat stronger binding is obtained when collagen III is labelled exclusively and therefore single staining would give better contrast compared to double staining.

### Fixation

All results from immunolabelling presented so far, were obtained on unfixed material with exception for fixation by heat treatment. It was found that fixation with acetone for ten minutes at room temperature or ethanol (several hours, -20 °C) gave no change in the degree of binding for Biogenex' antibody against actin. Paraformaldehyde treatment doubled the illumination from a labelled specimen of comminuted, raw meat compared to the same unfixed tissue. The morphology was, as expected, better for systems exposed to 4% paraformaldehyde followed by ethanol at -20 °C compared to no fixation as it was easier to see the transverse bands of the myofibrils corresponding to actin and myosin. The antibody against slow myosin bound more strongly to "free" myofibrils than to intact fibers as seen in Figure 9a. This was also the case for the unheated, fixed specimen seen in Figure 9c as well as for heat treated systems (results not shown). However, for fixed or heated tissue the difference in illumination between myofibrils in fibers and "free" myofibrils is less, as seen in Figure 9c. The antibody against actin did not reveal such a systematic differentiation between "free" myofibrils and myofibrils in intact fibers. In Figure 9b,

actin in intact fibers seems to be more strongly labelled than actin in "free" myofibrils while the fixed system in Figure 9d reveals no such preference for the antibody against actin.

## Discussion

### Species cross Reactivity

The majority of presently commercially available antibodies to muscle proteins have been raised using human molecules as immunogens. Accordingly, to employ a particular antibody to study meat from cows, pigs, sheep and other animals one has to rely on species cross reactivity. Muscle proteins from different species have highly conserved antigenic sites and species cross reactivity is therefore quite likely. It is nevertheless reassuring to use a second technique to confirm correct labelling. In this paper we have regarded ATP-ase and phalloidin staining as sufficiently reliable on fresh meat to check on species cross reactivity for antibodies against myosin and actin. For other proteins histological techniques might not be available or trustworthy. If the results from the immunolabelling seem dubious, it is recommended to confirm using electrophoretic techniques and immunoblotting even though these may appear less handy, at least in terms of instrumentation (Davis, 1964; Laemmli, 1970; Towbin *et al.*, 1979; Catty, 1988, 1989).

### Non-specific Staining

Non-specific staining which is not acknowledged as such, could be of great concern to scientists unfamiliar with immunohistology. Remedies for this are described in textbooks (Catty, 1988, 1989). However, there are extreme cases where it is difficult to eliminate the possibility of non-specific staining. This is to some extent the case for Sanbio's antibody against actin which bound to spots/filaments in the connective tissue (Figure 2c). Non-specific binding to the connective tissue is known to be an occasional problem (Bourne, 1983) and the antibody binding seen in Figure 2c therefore calls for some further investigations. The complementary technique which is sensitive to F-actin (Figure 2a), shows in general more binding to the connective tissue than is the case for the antibody. This is reassuring as actin is present in many cells and organelles including fibroblasts in the connective tissue (Ross *et al.*, 1989). The results shown in Figures 2c and 2d do therefore most likely reflect correct binding. Further doubts could be reduced by resorting to the same remedy as when checking for species cross reactivity (see above). The issue was, however, not pursued here, as no future, routine applications of this antibody are presently envisaged.



The reactivity, although weak, of the antibody directed against slow myosin with fast myosin becomes a complicating factor in comminuted meat products. It would then be difficult to differentiate between a smaller amount of slow myosin distributed evenly in the "continuous" phase from a larger amount of fast myosin also distributed evenly. However, a larger amount of slow myosin in the "continuous" phase should be recognized, especially since the binding to the liberated myofibrils is stronger than to the myofibrils in intact muscle cells. A second problem caused by incomplete discrimination, is related to the use of the antibody against slow myosin on muscles consisting of mainly fast fibers. If meat products are investigated following heat treatment to about 60 °C, incorrect information on the muscle type composition can easily be obtained as the binding pattern would reflect the heat denaturation of fast myosin. This is seen for *cutaneus trunci* in Figure 8. The antibody against slow myosin shows reduced affinity for heat denatured fast myosin and therefore less illumination from *cutaneus trunci* compared to *masseter*.

The reasons for the non-homogeneous staining of type I fibers by antibodies against slow myosin, as well as by ATP-ase staining (Figures 1b and 1a) are not clear. The non-homogeneous staining of type II fibers (Figure 1c) reflects the presence of fiber type IIA and type IIB.

#### Thermal Stability of Myosin, Collagen and Actin

The temperature ranges indicated in Figure 6 should be regarded as maximum temperatures except for actin whose denaturation is actually seen there. Previously reported thermograms were obtained by using higher heating rates than 1 °C/min which tends to shift the temperature ranges of denaturation to higher temperatures. Salt addition below 3% has limited effect on  $T_{end}$  for slow myosin at pH 6.0 (Egelandsdal *et al.*, submitted) and collagen denaturation (Judge and Aberle, 1982) while actin is greatly destabilized by NaCl addition as seen in Figure 6 and also reported by Stabursvik and Martens (1980). For the product containing 2.5% salt it is difficult to establish whether  $T_{end}$ , defined in Figure 6, reflects the end of thermal denaturation for actin or *perimysial* collagen. Results obtained on myofibrillar systems which show a better peak resolution, at comparable conditions (results not shown), suggest that actin would appear with  $T_{end}$  just below 72 °C and be more than 90% denatured at 70 °C.

Actin in *cutaneus trunci* denatures in practically the same temperature range as actin in *masseter* despite the pH difference. Only minor changes in the degree of denaturation for actin are therefore expected. Fast myosin has regions less stable and regions somewhat more stable (max. 4 °C) than slow myosin (Egelandsdal *et al.*, submitted).

#### Binding of Antibodies to Meat heat treated to different end Temperatures

The results obtained here on a limited number of commercial antibodies therefore suggest that it is possible to find preparations which are useful to apply to temperatures just above  $T_{end}$  as measured by DSC (Figure 6). Thermal denaturation is not necessarily leading to an extensive unfolding of the protein (Bertazzon *et al.*, 1990). It is therefore to be expected that antibody binding is possible to antigens heated to temperatures higher than  $T_{end}$  for a particular antigen.

The antibody against fast myosin could not be used to label up to  $T_{end}$ , as measured with a scanning calorimeter. The relevant epitope for the employed antibody might have low thermal stability. This particular antibody gave weak binding to our unheated, fresh systems reported in Figure 8, although good differentiation as seen in Figure 1a. The antibody against slow myosin is an antibody which was unexpectedly good and can in fact be used to temperatures 10-15 °C above  $T_{end}$  as measured with DSC. The antibodies against actin showed reduced binding at unexpectedly low temperatures, *i.e.*, above 50 °C. It is quite unlikely that actin has been heat denatured already at 60 °C as prolonged heating is needed to achieve denaturation of actin even at 65 °C (Martens *et al.*, 1982).

There is no reason to believe that increased temperature *per se* is a dominating factor causing reduced binding of antibodies upon heat treatment, however, it amplifies the effect of heat denaturation and this could be critical in a study of functional changes induced by denaturation of a particular heat stable antigen. The reason for reduced binding caused by increased temperature *per se*, can be related to changes in the surface properties of the object, caused by denaturation of less stable proteins than actin. Surface availability is of importance for the binding as demonstrated in Figure 9 using the antibody against slow myosin. Support for the idea that denaturation *per se* is not the only important factor causing reduced binding, can also be obtained by looking at Figure 4 where actin should be close to heat denatured for the systems containing 2.5% NaCl (panels 4b-4d).

The sample in Figure 3 has to be regarded as denatured above about 73 °C with respect to collagen III as well as slow myosin. The fate of *endomysial* collagen III was difficult to monitor for this system compared to the whole meat system seen in Figure 7c. In Figure 3b very weak binding is demonstrated to materials appearing in holes in the specimen, see arrow, and this binding might be to heat denatured collagen III which has melted.

Several commercial meat products are mildly heated to internal temperatures just above 68 °C (Ockerman,



1989). For such systems it should be possible to find good commercial antibodies to perform immunohistology. If immunohistology is a relevant technique for one particular problem, measures should be taken not to heat treat more powerfully than necessary with respect to establishing a relevant meat model. As it would be necessary to keep a strict time-temperature control in order to get sufficiently good binding for antibodies raised against the physiological structure of the antigen, the technique of immunohistology could yet be limited to research. Zijderveld and Koolmees (1990) reported only negative reactions when attempting immunolabelling on frozen sections of comminuted meat heated to 80 °C. Since no pictures were given of their systems, it is difficult to compare with our specimen heated to 80 °C and shown in Figures 1a, 1c and 7c. However, we feel it would be an exaggeration to report those reactions as negative. Zijderveld and Koolmees (1990) did not mention for how long their samples were heated at 80 °C and prolonged heating at 80 °C would be a disadvantage with respect to preserving relevant antigens. The somewhat better results reported by Zijderveld and Koolmees (1990) for the antigen actin present in comminuted meat embedded in paraffin sections, could be due to the use of formalin fixation for those samples. For food products which have received a large input of heat, such as autoclaving, we fully agree with the conclusion made by Zijderveld and Koolmees (1990) that useful antibodies for immunohistology must be raised against heat denatured antigens. However, since the number of commercial antibodies raised against the biological state of particular antigens are increasing, the possibilities for also finding antibodies useful at higher temperatures will increase.

#### Acknowledgements

Ina Storm is thanked for patience and skill during the printing of most of the photographs. Elvy Jordansson's assistance at the microscope is highly appreciated. Norwegian Council for Agriculture Research and MATFORSK, The Norwegian Food Research Institute, are warmly thanked for granting financial support for Bjørg Egelanddal's stay at SIK, The Swedish Food Research Institute, Gothenburg.

#### References

- Bailey A, Light ND. (1989). *Connective tissue in meat and meat products*. Elsevier Applied Sci., London, 120-147.
- Bernal VM, Stanley DW. (1987). Stability of bovine muscle connective tissue. *J. Food Sci.* **52**, 876-878.
- Bertazzon A, Tian GH, Lamblin A, Tsong TY. (1990). Enthalpic and entropic contributions to actin stability: calorimetric, circular dichroism, and fluorescence study and effects of calcium. *Biochemistry* **29**, 291-298.
- Bourne JA. (1983). *Handbook of immunoperoxidase staining methods*, Dako Corp., Santa Barbara, Calif., 13-19.
- Brooke MH, Kaiser KK. (1970). Muscle fiber types: How many and what kind? *Arch. Neurol.* **23**, 369-379.
- Catty D. (1988). *Antibodies: A practical approach*, vol I, IRL Press, Oxford, 9, 76-78, 137-169.
- Catty D. (1989). *Antibodies: A practical approach*, vol II, IRL Press, Oxford, 97-155, 175-176.
- Davis BJ. (1964). Disc electrophoresis II. Method and application to human serum albumin protein. *Ann. N. Y. Acad. Sci.* **2**, 404-427.
- Fakan F, Saskova O. (1982). Distribution of pseudo-elastica and elastin in some human tissues during postnatal life. A light microscopic study. *Folia morphologica* **30**, 64-69.
- Judge MD, Aberle ED. (1982). Effects of chronological age and postmortem aging on thermal shrinkage temperature of bovine intramuscular collagen. *J. Anim. Sci.* **54**, 68-71.
- Keith DA, Paz MA, Gallop PM, Glimcher MI. (1977). Histologic and biochemical identification and characterization of an elastin in cartilage. *J. Histochem. Cytochem.* **25**, 1154-1162.
- Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-682.
- Martens H, Stabursvik E, Martens M. (1982). Texture and color changes in meat during cooking related to thermal denaturation of muscle proteins. *J. Tex. Stud.* **13**, 291-309.
- Meyer C, Egelanddal B. (1992) Low deformation and water holding measurements on whole and comminuted meat originating from two muscles of different fiber type composition. *J. Tex. Stud.* **23**(1), in press.
- Ockerman HW. (1989). *Sausage and processed meat formulations*. Van Nostrand Reinhold, 15-562.
- Pierard GE. (1989). Sirius red polarization method is useful to visualize the organization of connective tissues but not the molecular composition of their fibrous polymers. *Matrix* **9**, 68-71.
- Rao MV, Gault NFS. (1989). The influence of fibre-type composition and associated biochemical characteristics on the acid buffering capacities of several beef muscles. *Meat Sci.* **26**, 5-17.
- Rochdi A, Bonnet M, Kopp J. (1985). Denaturation du collagène musculaire au cours du chauffage (Denaturation of muscle collagen during heating. Effect of restraint of fibers on shrinkage and degree of denaturation). *Sci. Aliments* **5**, 293-298.



Romeis B. (1989). Untersuchung des Binde- und Stützgewebes (Investigation of connective Tissue). In: Mikroskopische Technik, Börk P (Ed.), Urban and Schwarzenberg, München, chapter 25, 517-522.

Ross MH, Leith EJ, Romrell EJ. (1989). Histology: A text and atlas, 2nd ed., Williams and Wilkins, Baltimore, 216-224.

Stabursvik E, Martens H. (1980). Thermal denaturation of proteins in post-rigor muscle tissue as studied by dsc. J. Sci. Food Agric. **31**, 1034-1042.

Steven A. (1982). Enzyme histochemistry: diagnostic applications. In: Theory and Practice of Histological Techniques, Bancroft JD, Stevens A (Eds.), Churchill Livingstone, London, chapter 19, 400-411.

Sweat F, Pichtler H, Rosenthal, SI. (1964). Sirius red F3BA as stain for connective tissue. Arch. Patol. **78**, 69-72.

Tang X, Lancelle SA, Hepler PK. (1989). Fluorescence microscopic localization of actin in pollen tubes: Comparison of actin anti-body and phalloidin staining. Cell Mot. Cytoskel. **12**, 216-224

Towbin H, Staehelin T, Gordon J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76**, 4350-4354.

Young OA, Davey CL. (1981). Electrophoretic analysis of proteins from single bovine muscle fibers. Biochem. J. **195**, 317-327.

Zijderveld MHG, Koolmees PA. (1990). Utility of immunohistochemical identification of muscle proteins in microstructural studies of comminuted meat products. Meat Sci. **27**, 55-60.

#### Discussion with Reviewers.

**G. Kraal:** Would it not be better, for the general use of the method, to develop antibodies which are species-specific?

**Authors:** Species-specific antibodies would be, generally speaking, preferable. However, *a priori* some cross reactivity should be expected and on that background the ultimate purpose of a test for a particular antigen should determine the need for developing species-specific antibodies. If, for example, the question is whether beef and pork myosins in a mixture behave differently, species-specific antimyosins would be needed. On the other hand if information on all myosins would be more rewarding, a non species-specific, commercial, polyclonal antibody should be adequate and cheaper to use.

**G. Kraal:** Why did the authors switch to pork when studying a commercial food specimen? Are the controls and staining specificities studied for beef valid for pork?

**Authors:** In the study of beef reported here, we have examined practically all commercial conditions except chilled storage for more than four days. It was therefore felt that to present the beef system also as Figure 3 would be superfluous. We have assured ourselves of the specificities of the antibodies used in Figure 3 for the pork system. However, controls could not be obtained on the particular specimen shown in Figure 3, since it was received *post* heated.

**P. Wilding:** The authors estimate the extent of protein denaturation by comparison of heating temperatures with temperatures on a DSC thermogram. Would not a better approach have been to analyze the samples in a calorimeter and identify directly the proteins that were denatured previously?

**Authors:** We understand the question to imply that previously heated meat samples should be subjected both to immunolabelling and calorimetric examinations. This is an equally good approach provided more sections (samples) than 5-6 (see legends for Figure 8) were used at each temperature. If not, it would be more difficult to compare the figures obtained for illumination at different temperatures as the surface characteristics of a particular section affect the ultimate illumination measured.

**P.A. Koolmees:** The identification in comminuted meat (Figures 3 and 9) is less distinct compared to intact muscle tissue. Would you expect to obtain more detailed information about functional properties of meat protein using these techniques in addition to the examination of sections from comminuted, salted and heat treated meats stained with for instance toluidine blue or Sirius red F3BA, especially at higher magnification?

**Authors:** We have compared labelling with antibodies against collagen both with staining with Sirius red and a modified technique for Aniline blue and Orange G. Both staining techniques are qualitative. We find the staining with Sirius red is less reliable than the immunostaining because of the tendency of overstaining for collagen.

**P.A. Koolmees:** Zijderveld and Koolmees (1990) reported that the reactivity of intact muscle tissue decreased as the tissue was more intensively denatured by comminution or heating. Based on the results reported here, do you think immunolabelling provides possibilities for localizing specific meat proteins in comminuted meat products?

**Authors:** The localization of specific meat proteins in unheated, comminuted meat products should not present a problem. We have not observed that comminution *per se* reduces the illumination from the immunostained tissue compared to the intact tissue. As regards heat



input strongly influences the reactivity seen. However, Zijderveld and Koolmees (1990) and we have all looked at only a few of the commercial antibodies available against for example actin and based our conclusions on that. To analyze any commercial meat product a mixture of antibodies raised against the biological state, and the strongly heated state, would be needed for reliable staining. Unfortunately, the availability of antibodies against antigens which have received a strong heat input, in particular species-specific antibodies, will probably be poor for years.

**R. Cassens:** What would the use of the techniques add to our already existing knowledge of structure of muscle and meat? Is there any practical application for the industry?

**Authors:** With respect to genetic variants of different proteins there is presently only some understanding about how different myosin isoforms and collagen III affect meat functionality or texture. With respect to the rest of the genetic variants, contributions to functionality have to be revealed.

It is presently possible, and of interest, to look at the distribution of different genetic variants of a protein in a comminuted, unheated or mildly heat treated, meat products aiming at elucidating their possibilities for network formation. The effect of salt addition on different genetic variants can also be studied *in situ*. We have, for example, been able to locate pools of salt-solubilized myosin or actin in non-comminuted meat, and looked at the prevalence of such pools in different muscles (results not shown). Eventually that type of information is of use to the industry. However, perhaps the most straight forward practical application presently is to study the distribution of proteinaceous additives in meat products as this will elucidate both the efficiency of the mixing process, and the functional performance of the additives. In order to obtain such information harsh heat treatment is not necessarily a prerequisite.

In addition, more detailed understanding about the structure of thick filaments and myosin has in recent years been obtained by using monoclonal antibodies and electron microscopy (Shimizu *et al.*, 1985; Tokunaga *et al.*, 1987). The location of new proteins in the skeletal muscle has also been established using monoclonal antibodies at the level of electron and light microscopy. The distribution of isoforms of C-protein in skeletal muscle has been given using immunostaining and light microscopy (Dhoot *et al.*, 1985). Similar details on muscle structure, demonstrated by immunostaining, is likely to be revealed still. An important question is, however, how this more detailed information on the structural organization of muscle contributes to our understanding of meat functionality. We think that the answer to that question belongs to the future.

#### Additional References

Dhoot GK, Hales MC, Grail BM, Perry, SV. (1985). The isoforms of C-protein and their distribution in mammalian skeletal muscle. *J. Muscle Res. Cell Motil.* **6**, 487-505.

Shimizu T, Dennis JE, Masaki T, Fischman, DA. (1985). Axial arrangement of the myosin rod in vertebrate thick filaments: Immunoelectron microscopy with a monoclonal antibody to light meromyosin. *J. Cell Biol.* **101**, 1115-1123.

Tokunaga M, Suzuki M, Saeki K, Wakabayashi T. (1987). Position of the amino terminus of myosin light chain 1 and light chain 2 determined by electron microscopy with monoclonal antibody. *J. Mol. Biol.* **194**, 245-255.